

BRIEF REPORT

Thrombotic potential during pediatric acute lymphoblastic leukemia induction: Role of cell-free DNA

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Abstract

Background: Thromboembolism affects up to 30% of children undergoing treatment for acute lymphoblastic leukemia (ALL). Increased thrombin generation has been reported in ALL, but the mechanisms remain elusive.

Objective: We aimed to show that extracellular traps and cell-free DNA (cfDNA) promote thrombin generation in pediatric ALL.

Methods: In a longitudinal single-center study, we recruited 17 consecutive pediatric ALL patients. Serial blood samples were collected at diagnosis and weekly during the 4-week induction phase of antileukemic chemotherapy. Healthy children (n = 14) and children with deep vein thrombosis (DVT; n = 7) or sepsis (n = 5) were recruited as negative and positive controls, respectively. In plasma, we measured endogenous thrombin generation potential (ETP) and components of extracellular traps, including cfDNA.

Results: In patients with ALL, ETP was increased at baseline and remained significantly elevated throughout the induction therapy. Plasma levels of cfDNA were increased at baseline and during the first 3 weeks of induction therapy. The extent of enhancement of ETP and plasma cfDNA in patients with ALL was similar to that seen in patients with DVT or sepsis. Treatment of plasma with DNase 1 lowered ETP in patients with ALL at each time point but did not affect ETP in healthy controls.

Conclusion: We conclude that childhood ALL is associated with a prothrombotic milieu at the time of diagnosis that continues during induction chemotherapy, and cfDNA contributes to increased thrombogenic potential.

KEYWORDS

cell-free nucleic acids, child, extracellular traps, induction chemotherapy, leukemia, thrombin generation

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Essentials

- Thromboembolism affects $\leq 30\%$ of children undergoing treatment for acute lymphoblastic leukemia (ALL).
- We hypothesized that extracellular traps and cell-free DNA increase thrombin generation in these patients.
- We found that thrombin generation potential was increased before and during chemotherapy.
- Treatment of plasma with DNase 1 lowered thrombin generation potential.

1 | INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common malignancy in childhood, affecting about 30 per million children.¹ Complications associated with ALL and its therapy remain a challenge to successful outcomes. Thromboembolism in particular may affect up to 30% of children undergoing treatment for ALL.² A majority of thrombotic complications occur during the first 4 weeks of therapy, known as the “induction” phase, during which patients receive intensive anti-leukemic therapy to induce remission.

Several therapy-related hemostatic alterations occur with ALL that have the potential to cause hypercoagulability.^{3,4} Increased thrombin generation has been reported in pediatric ALL,^{4–6} but the mechanisms remain elusive. Recent studies have focused on the role of extracellular traps, which contain decondensed chromatin nucleosomes (histone-bound DNA), citrullinated histones, and cell-free DNA (cfDNA) as potential mediators of increased thrombin generation in a variety of clinical settings associated with thrombosis.^{7–9} Most studies have characterized extracellular traps released from neutrophils, but other hematopoietic cells such as eosinophils,¹⁰ mast cells,¹¹ and basophils¹² can also contribute to their formation. It is possible that blast cells undergoing lysis during therapy phase may likewise release cfDNA or citrullinated histones. We therefore hypothesized that increased thrombogenic potential in children undergoing induction therapy for ALL may be driven by release of cfDNA or other components of extracellular traps.

To address this question, we performed a longitudinal study in pediatric patients with ALL. The objectives were to determine if components of extracellular traps are elevated in the plasma of children with ALL and to define the contribution of cfDNA to enhanced thrombin generation potential.

2 | METHODS

We designed a longitudinal single-center cohort study of consecutive pediatric patients with ALL undergoing induction with anti-leukemic therapy at the Stead Family Children's Hospital (SFCH) at the University of Iowa from 2016 through 2018. We recruited a total of 17 children with ALL and 28 additional subjects: 7 children with sepsis, 5 with deep vein thrombosis (DVT), and 14 healthy controls. Healthy children without any acute or chronic illnesses, and children with sepsis or DVT were recruited through the outpatient clinics and inpatient units of the SFCH. The protocol was approved by the University of Iowa Institutional Review Board (IRB). Informed consent and assent were obtained from parents/guardians of minor

children and children >7 years of age, respectively. Assent was waived for critically ill children.

ALL was diagnosed using standard World Health Organization criteria with $>20\%$ of lymphoblasts in blood or bone marrow and lymphoblast subtyping by flow cytometry.¹³ Patients with ALL were categorized on the basis of lymphoblast phenotype and National Cancer Institute risk stratification as T-cell ALL ($N = 3$); pre-B-cell ALL, standard risk (SR; $N = 10$); or pre-B-cell ALL, high risk (HR; $N = 4$). Subjects were followed longitudinally from diagnosis through completion of 4 weeks of induction chemotherapy according to Children's Oncology Group protocols that consisted of a three (for pre-B SR) or four (for pre-B HR or T-cell ALL) drug regimen with corticosteroids, vincristine, pegylated asparaginase, and daunorubicin (only for four drug regimens). None of the subjects received anticoagulants or antiplatelet therapy.

2.1 | Sample collection and processing

For patients with ALL, blood samples were collected at the time of diagnosis (baseline) and weekly during the 4-week induction therapy time period (a total of five time points). The baseline sample was collected within 24 hours before initiation of induction chemotherapy. For control subjects, a blood sample was collected once only. Whole blood was collected into tubes containing 3.2% Na citrate through a central venous line (CVL) for patients with ALL and through an antecubital vein from control subjects. All samples were collected in the morning and processed within 30 minutes. Blood was centrifuged at 2500 g for 10 minutes at room temperature. The supernatant fraction of platelet-poor plasma (PPP) was transferred to a separate tube and centrifuged again at 10 000 g for 10 minutes and aliquoted and stored at -80°C until assays were performed. The volume of blood and sample collection complied with IRB guidelines.

2.2 | Thrombin generation

Thrombin generation was measured using the Calibrated Automated Thrombogram (CAT, Diagnostica Stago, Inc, Parsippany, NJ, USA).¹⁴ Briefly, 80 μL of PPP was incubated with 20 μL “PPP low” reagent containing 1 pM of tissue factor and phospholipids for 10 minutes, followed by addition of a fluorogenic substrate containing CaCl_2 . Thrombin generation was measured continuously, and parameters of lag time, peak thrombin, and endogenous thrombin potential (ETP, a measure of the area under the thrombin generation curve)

were recorded. In experiments with DNase 1, PPP samples were treated with 20 µg/mL of DNase 1 (Worthington Biochemical Corp., Lakewood, NJ, USA) or heat-inactivated DNase 1 (control) at 37°C for 60 minutes prior to assay.

2.3 | Components of extracellular traps

Quantification of circulating cfDNA in plasma samples was performed using the Qubit dsDNA HS Assay (Invitrogen, Life Technologies, Carlsbad, CA, USA). Levels of citrullinated histone H3 (H3Cit) were measured by ELISA (Clone 11D3, Cayman Chemical, Ann Arbor, MI, USA), and nucleosomes were measured using the Cell Death Detection ELISA (Roche Diagnostics, Basel, Switzerland), which detects mono- and oligonucleosomes, and presented as fold change compared to healthy controls.

2.4 | Other hematologic assays

Complete blood cell count was performed using Sysmex XN-450/XN-430, a quantitative automated hematology analyzer. Plasma prothrombin and serum plasminogen activator inhibitor-1 (PAI-1) were measured using human-specific ELISAs (Molecular Innovations, Southfield, MI, USA).

2.5 | Statistical analysis

Prism software (GraphPad Software, La Jolla, CA, USA) was used for all statistical analysis. Descriptive analyses were performed using mean, median, and standard deviation for the age and blood cell counts. A normality test was performed on each data set using the D'Agostino-Pearson omnibus K2 test. For parameters showing a nonlinear distribution, the Kruskal-Wallis test on ranks was performed. Dunn's test was used for multiple comparisons. One-way or two-way analysis of variance with Tukey's test for multiple comparisons was performed to analyze data sets that showed normal distribution. In studies using DNase 1 treatments, the two-stage linear step-up procedure of Benjamini, Kreiger, and Yekutieli was performed. Statistical significance was defined as $P < .05$.

3 | RESULTS AND DISCUSSION

Detailed patient characteristics and hematologic parameters are provided in Table 1. The ETP was significantly elevated during weeks 1 to 4 of induction therapy in patients with ALL and in patients with DVT ($P < .05$ and $P < .01$ vs healthy controls, respectively) (Figure 1A). No differences in lag time to initial thrombin generation were observed between healthy controls, patients with ALL at baseline or during induction therapy, or children with DVT

TABLE 1 Hematologic parameters for healthy controls and patients with ALL

	Healthy controls (N = 16)	Patients with ALL (N = 17)				
		Baseline, week 0 ^a	Week 1	Week 2	Week 3	Week 4
Age, y	5.0 (0.5-17)	5.9 (1.5-14.2)				
Sex, M:F	6:8	10:7				
Hemoglobin, g/dL	12.3 ± 0.2	8.1 ± 0.4*	9 ± 0.4*	8.3 ± 0.3*	8.7 ± 0.3*	8.8 ± 0.3*
Hematocrit, %	37.1 ± 0.8	23.5 ± 1.3*	25 ± 1*	23.7 ± 0.8*	25.6 ± 0.8*	26.7 ± 0.8*
Platelet count, mean, ×10 ³ /µL	360 ± 21	74 ± 13*	62 ± 14*	105 ± 20*	197 ± 31 ^{*,*****}	241 ± 15 ^{*,*****}
WBC, mean, ×10 ³ /µL	6.4 (1.8-10.2)	9.5 (1.8-374)	2.2 ^{***} (0.3-22.4)	0.9 ^{***} (0.2-2.5)	2.0 ^{***} (0.3-19.3)	5.4 ^{*,*****} (1.2-11.6)
Neutrophils, ×10 ³ /µL	2240 (980-7830)	138* (0-14 980)	210* (100-3810)	720 (73-1175)	1280 (50-4030)	2816 ^{*,*****} (120-5308)
Lymphocytes, ×10 ³ /µL, check stat with median	3420 ± 415	9243 ± 5081	1046 ± 501 ^{***}	573 ± 107 ^{***}	891 ± 205 ^{***}	1382 ± 235
Monocytes, ×10 ³ /µL	460 (220-900)	100 (20-1350)	20* (0-670)	46* (7-470)	160 (32-3090)	328 ^{*,*****} (46-4140)
Blasts, ×10 ³ /µL	0	5* (0-278)	0	0	0	0
Plasma prothrombin, µg/mL	206 ± 24	216 ± 68.4	226 ± 23.2	227 ± 23.2	222 ± 14.5	ND
Serum PAI-1, ng/mL	38.8 ± 1.4	21.3 ± 3.6*	17.8 ± 3*	28.2 ± 3.4	37.1 ± 2.4 ^{*,*****}	ND

Note: Data sets showing Gaussian distribution are presented as Mean ± standard error and were analyzed using one-way analysis of variance with Tukey's test for multiple comparisons. Data sets showing a non-Gaussian distribution are presented as median (range) and were analyzed using the Kruskal-Wallis test with Dunn's test for multiple comparisons.

Abbreviations: ALL, acute lymphoblastic leukemia; ND, not done; PAI-1, plasminogen activator inhibitor-1; WBC, white blood cell count.

^aWeek 0 baseline sample was collected within 24 h before starting the chemotherapy.

* $P < .05$ vs healthy controls; ** $P < .05$ vs baseline; *** $P < .05$ vs week 1 of induction therapy; **** $P < .05$ vs week 2 of induction therapy.

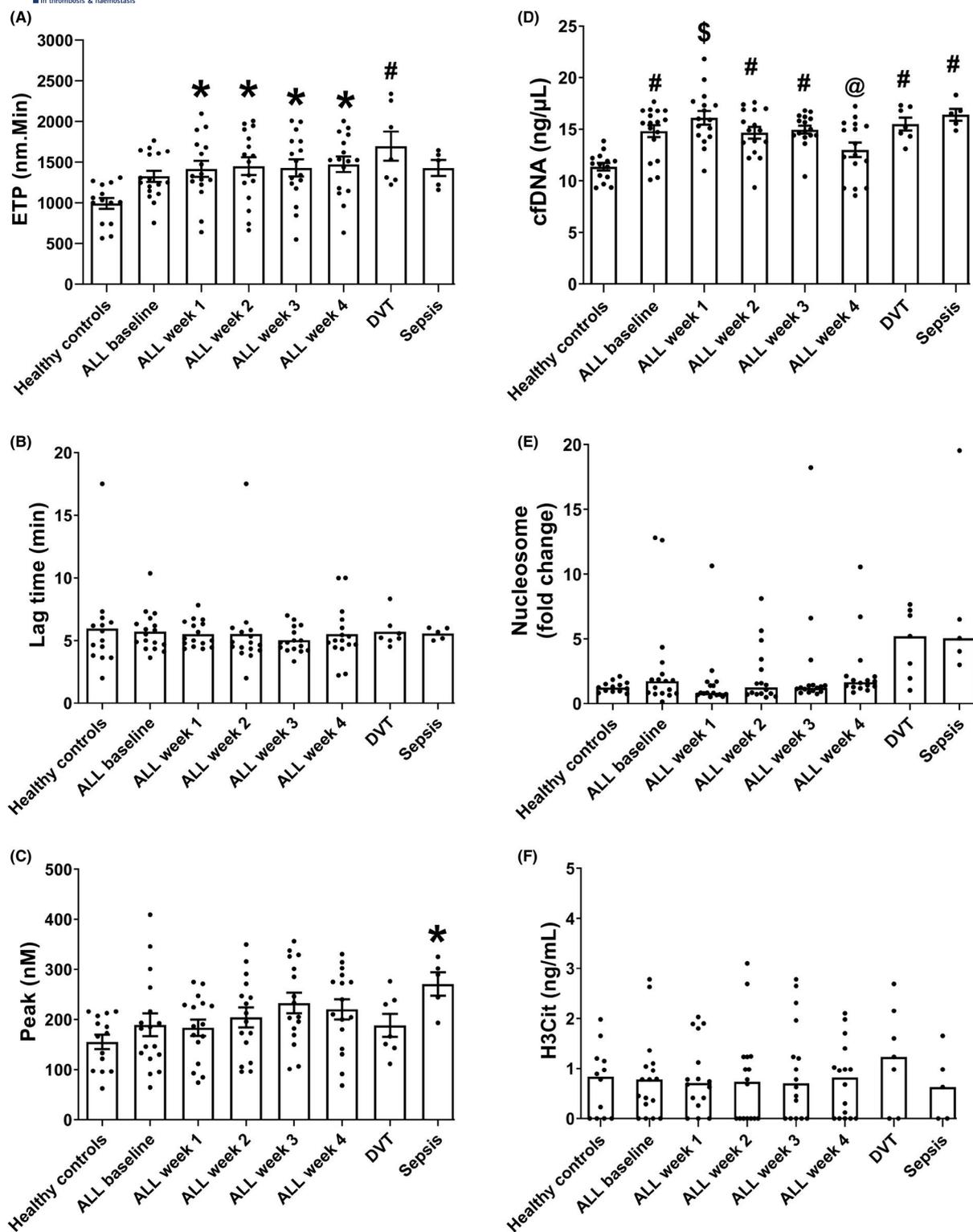


FIGURE 1 Persistence of increased endogenous thrombin potential and elevated plasma cell-free DNA (cfDNA) levels during induction chemotherapy in patients with acute lymphoblastic leukemia (ALL). Thrombin generation potential and components of extracellular traps were measured in plasma samples from healthy pediatric controls and patients with deep vein thrombosis (DVT), sepsis, or ALL at diagnosis (baseline) and during 1 to 4 weeks of induction chemotherapy. (A) Endogenous thrombin potential (ETP); (B) lag time; (C) peak thrombin generation; (D) cfDNA. (E) nucleosomes; (F) citrullinated histone H3 (H3Cit). One-way analysis of variance with Tukey's test for multiple comparisons was used to analyze ETP, peak thrombin and cfDNA and individual data points are presented as well as mean \pm standard error. Data for lag time, nucleosome, and H3Cit are presented as median and were analyzed using the Kruskal-Wallis test with Dunn's test for multiple comparisons. * $P < .05$; # $P < .01$, and \$ $P < .0001$ vs healthy controls and @ $P < .05$ vs week 1

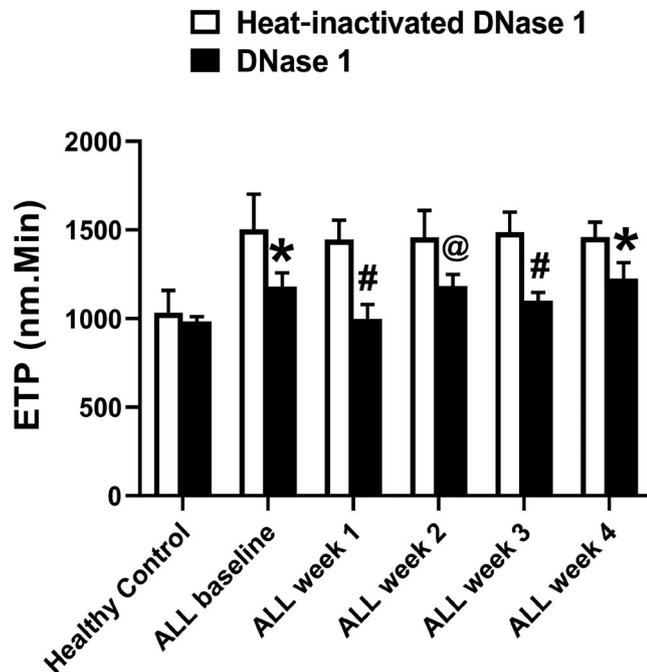


FIGURE 2 Increased thrombin generation potential in patients with acute lymphoblastic leukemia (ALL) is mediated through cfDNA. Platelet-poor plasma collected from the patients at baseline or during 1 to 4 weeks of induction therapy was treated with 20 μ g of DNase 1 (Worthington Biochemicals) or heat-inactivated DNase 1 (control) at 37°C for 60 min prior to assay, following which thrombin generation was measured. Endogenous thrombin potential (ETP) is presented as mean \pm standard error. N = 6. * $P < .05$, @ $P < .01$, and # $P < .0001$ vs heat-inactivated DNase 1 at respective time points, analyzed using multiple t test with two-stage linear step-up procedure

or sepsis (Figure 1B,C). Peak thrombin was significantly higher than healthy controls in patients with sepsis ($P < .05$) but not in patients with ALL or DVT (Figure 1C). The observation that ETP was increased in ALL is consistent with previous observations that indirect markers of thrombin generation, such as thrombin-antithrombin complexes, are elevated in the plasma of patients with ALL at diagnosis and during treatment.^{3,4} The enhanced thrombin generation potential in ALL cannot be attributed to changes in plasma prothrombin concentration, which was similar in all patient groups (Table 1). Unlike some prior studies in which elevated levels of PAI-1 were reported in children with ALL,^{3,4} we found that patients with ALL had 50% lower plasma levels of PAI-1 at baseline compared to healthy controls, and that PAI-1 increased toward normal during induction therapy, reaching control levels by week 3 (Table 1). This suggests that elevation of PAI-1 is not mediating the increase in ETP.

We next measured plasma levels of components of extracellular traps, including cfDNA, nucleosomes, and H3Cit, which have been reported to be elevated in humans with some types of cancer.¹⁵⁻¹⁷ The prevalence of these biomarkers and their contribution to thrombogenesis in childhood ALL is not known. cfDNA is known to promote blood coagulation via activation of factor XII, and histones

may promote thrombosis indirectly by activating platelets or inhibiting thrombomodulin-mediated protein C activation.^{7,18} We found that, compared with healthy controls, patients with ALL had significantly higher plasma levels of cfDNA at baseline and during the first 3 weeks of induction therapy, with the highest levels observed at week 1 ($P < .0001$) (Figure 1D). By week 4, plasma concentrations of cfDNA declined to levels similar to those in healthy controls and were significantly lower than at week 1 ($P < .05$). Levels of plasma cfDNA also were significantly elevated in patients with DVT or sepsis when compared to healthy controls ($P < .01$) (Figure 1D). In contrast, plasma levels of nucleosomes (Figure 1E) and H3Cit (Figure 1F) did not differ significantly between healthy controls and patients with ALL at any time point. We also did not observe significant elevations of nucleosomes or H3Cit in children with DVT or sepsis. Our findings of elevation in plasma cfDNA, but not nucleosomes or H3Cit, may suggest that circulating cfDNA in patients with ALL may arise not only from extracellular traps but also from apoptosis or necrosis of leukemic blasts or other cells.

To define the mechanistic role of cfDNA in mediating enhanced thrombin generation potential in patients with ALL, we treated plasma samples with DNase 1 prior to measuring thrombin generation. In samples from healthy controls, treatment with DNase 1 did not affect ETP (Figure 2). In contrast, in samples from patients with ALL, treatment with DNase 1 decreased ETP significantly at baseline and also throughout induction therapy, indicating that much of the ETP is mediated by prothrombotic DNA. Our findings are in agreement with prior work, demonstrating that the prothrombotic phenotype in certain animal models of cancer can be reversed by treatment with DNase 1.¹⁹⁻²¹

We also examined the relationship between cfDNA, ETP, and peripheral white blood cell counts during the 4 weeks of induction therapy (Figure 3A,B). As expected, peripheral blast cells disappeared by week 1 and remained undetectable thereafter. Neutrophils, monocytes, and lymphocytes decreased during the first 2 weeks and then increased to near normal counts by week 4. In contrast, cfDNA remained significantly elevated for 3 weeks before declining modestly at week 4 (Figure 3A), and ETP was elevated at all time points (Figure 3B). Thus, we did not observe a direct correlation between peripheral blast cell counts and cfDNA or thrombin generation and it remains uncertain whether the elevated cfDNA in patients with ALL was derived from neutrophils, lymphoblasts, or other cells such as endothelial cells. We also did not observe significant differences in the time course of plasma cfDNA or ETP between patients with different subtypes of ALL (Figure 3C-H), although patients with T-cell ALL had slightly higher levels of cfDNA at week 1 compared to patients with pre-B ALL (Figure 3C).

One limitation of our study is that the relatively small sample size limited our ability to assess associations between thrombin generation, cfDNA, and clinical thrombosis. None of our ALL patients developed a clinically significant thrombotic event during the 4-week induction phase of therapy, and only three developed CVL malfunction, which can be a surrogate of underlying venous thrombosis.²²⁻²⁴ We could not rule out subclinical thrombosis since surveillance

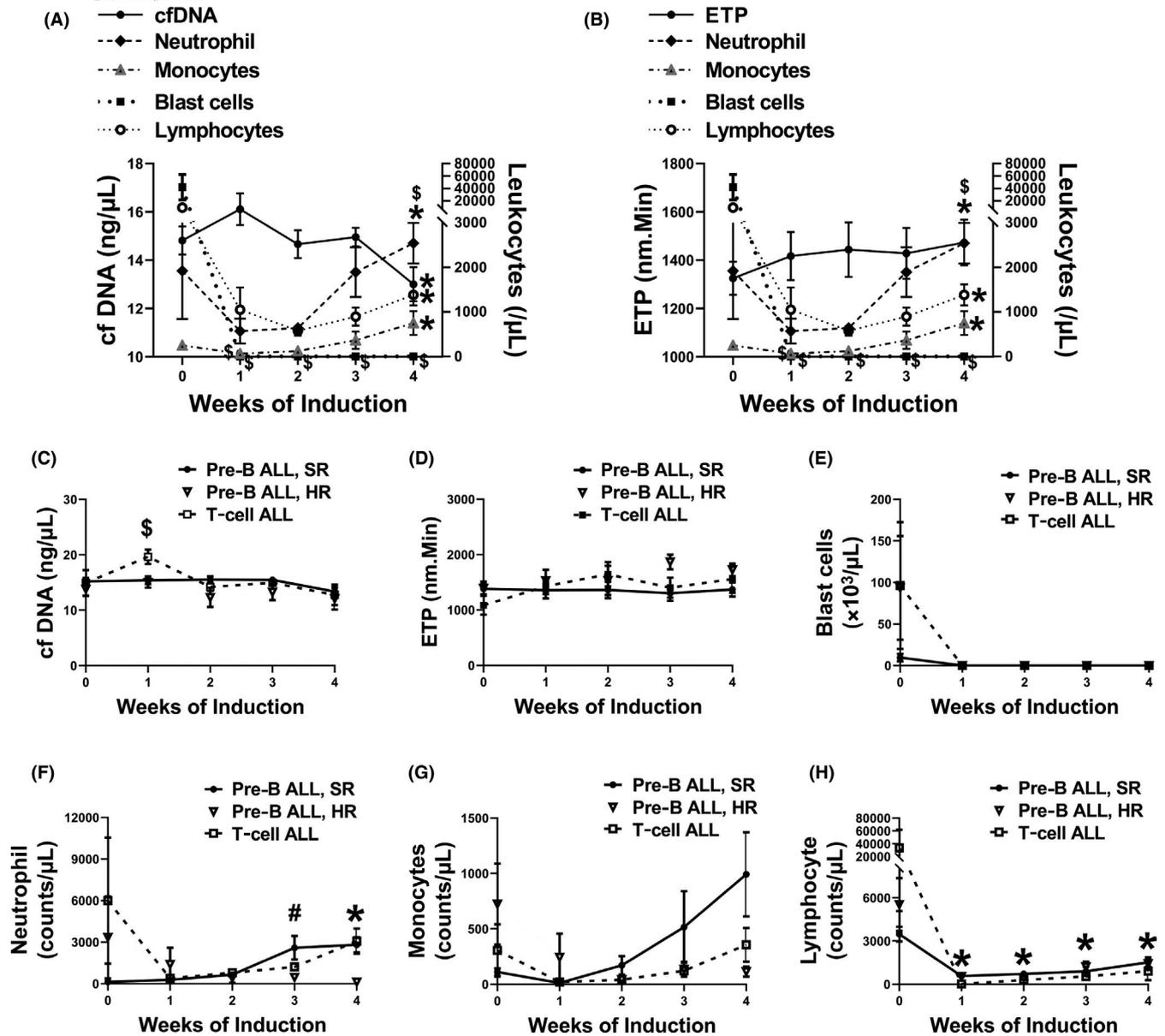


FIGURE 3 Pattern of cellular changes during induction therapy in relation to plasma cell-free DNA (cfDNA) levels and thrombin generation potential and acute lymphoblastic leukemia (ALL) subtypes. Weekly changes in leukocyte counts in relation to plasma cfDNA (A) and endogenous thrombin potential (ETP) (B) is depicted as line graph (Mean \pm standard error) using dual Y-axis plot. * $P < .05$ vs week 1 and $^{\$}P < .05$ vs baseline. Two-way analysis of variance, mixed effects with Tukey's analysis. (C-H) Weekly changes in plasma cfDNA, ETP, blast cells, neutrophils, monocytes, and lymphocytes in ALL subtypes: pre-B ALL, standard risk (SR; $N = 10$); pre-B ALL, high risk (HR; $N = 4$); and T-cell ALL ($N = 3$), depicted as line graph (mean \pm standard error). * $P < .01$ vs baseline for pre-B ALL, SR, # $P < .05$ vs week 1 for pre-B SR, and $^{\$}P < .05$ vs pre-B SR and pre-B HR at week 1. Two-way analysis of variance with Tukey's analysis

ultrasound or venography was not done.² Therefore, we were unable to assess relationships between thrombin generation potential or biomarkers with symptomatic or asymptomatic thrombosis. We also did not address the potential role of platelets in the prothrombotic milieu during ALL induction therapy. It is possible that platelet activation and microvesicle formation may contribute to thrombin generation and that this effect might vary during induction therapy as patients become thrombocytopenic. Another limitation could be that we measured PAI-1 in serum that may contribute to an artifactual elevation due to platelet activation and thus limited the ability

to detect associations with thrombin generation. Future studies are needed to address the clinical correlations of ex vivo thrombin generation, platelet-derived microvesicles, and other thrombotic biomarkers in this patient population.²⁵⁻²⁷

In summary, our findings demonstrate that a prothrombotic state manifested by increased thrombin generation potential exists in pediatric patients with ALL at baseline and throughout the induction phase of chemotherapy. Importantly, we found that the increased thrombogenic potential can be reversed by treatment with DNase 1, which suggests a direct mechanistic pathway and a potential

approach to prevention and treatment of thrombotic complications in patients with ALL. This approach may prove to be feasible in the clinic, since DNase 1 is also under development as a therapeutic approach in other conditions such as cystic fibrosis,²⁸ Alzheimer disease,²⁹ and coronavirus disease 2019.³⁰ Our findings also raise some interesting mechanistic questions for future study, including:

1. Is the protective effect of DNase 1 on thrombin generation mediated by nucleolytic degradation of both cfDNA and histone-bound DNA (both of which are known to have prothrombotic properties³¹)?
2. What are the cellular sources of elevated cfDNA in patients with ALL?
3. How much cfDNA is derived from nuclear chromatin versus mitochondrial DNA?

It also will be interesting in future studies to measure cfDNA and thrombin generation during subsequent postinduction courses of ALL chemotherapy, since it is likely that a subset of patients may remain at risk for thrombotic episodes after induction therapy is completed.

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RELATIONSHIP DISCLOSURE

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

RK designed and conducted the experiments, interpreted the results, and cowrote the manuscript. PBK also conducted the experiments. SLR assisted with the design and interpretation of the results. AJM assisted with patient recruitment and interpretation of the results. AAS participated in study design, subject recruitment, helped setting up thrombin generation assay and data interpretation. SD directed the project, designed the experiments, interpreted the results, and wrote the manuscript. All authors assisted with the preparation and editing of the manuscript.

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